



Exploitation or cooperation? Evolution of a host (ciliate)-benefiting alga in a long-term experimental microcosm culture



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ARTICLE INFO

Article history:

Received 11 March 2013
Received in revised form 28 May 2013
Accepted 30 May 2013

Keywords:

Endosymbiosis
Evolution
Mixotrophy
Cooperation
Tetrahymena
Micractinium

ABSTRACT

Controversy persists as to whether the acquisition of beneficial metabolic functions via endosymbiosis can occur suddenly on an evolutionary time scale. In this study, an early stage of endosymbiotic associations, which evolved from previously unassociated auto (photo)- and heterotrophic unicellular organisms was analyzed using an experimental ecosystem model, called CET microcosm. This ecosystem model was composed of a green alga (*Micractinium* sp.; formerly described as *Chlorella vulgaris*), a bacterium (*Escherichia coli*), and a ciliate (*Tetrahymena thermophila*). Our previous study using a CET microcosm that was cultured 3–5 years revealed that fitness of the ciliate increased by harboring algal cells within its own cells. This fact suggested three possibilities: (i) the ciliate evolved the ability to exploit intracellular algal cells (“exploiter ciliate hypothesis”), (ii) the alga evolved the ability to benefit the host ciliate by providing photosynthates (“cooperator alga hypothesis”), and (iii) a combination of (i) and (ii). To test these hypotheses, two-by-two co-cultures were conducted between the ancestral or derived ciliate and the ancestral or derived alga. The experimental results demonstrated that a cooperative alga evolved in the microcosm, although the possibility remains that an exploitative genotype of the ciliate might also exist in the population as a polymorphism. Remarkably, an algal isolate prolonged the longevity of not only the isolated ciliate, but also the ancestral ciliate. This result suggests that once a cooperative algal genotype evolves in a local population, it can then be transmitted to other individuals of the prospective host species and spread rapidly beyond the local range due to its positive effect on the host fitness. Such transmission suggests the possibility of a sudden acquisition of beneficial autotrophic function by the pre-associated host.

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1. Introduction

Endosymbiosis results in a nested symbiont-within-host cell structure, which combines different metabolic functions that evolved separately in the different species. Consequently, such associations can result in innovative adaptations, enabling the organism to persist in an environment that each species would not otherwise be able to survive and reproduce in on its own (Margulis, 1993). Endosymbiosis can originate from various forms of non-symbiotic relationships, such as parasitism/pathogenesis, predation, including active and passive uptakes, and lateral transmission of symbionts that are already adapted to other host species (Douglas, 2010). It has been suggested that symbiogenesis, i.e., the evolution of adaptations by symbiosis, may challenge the Darwin's theory as the sole, explanatory framework for adaptive evolution in the living world, because adaptive traits of partners could

be acquired by a non-gradual process (Khakhina, 1992; Margulis, 1993; Ryan, 2002; Roossinck, 2005; Watson, 2006).

However, endosymbiosis often involves conflict between the host and the symbiont. According to virulence theory, this relationship may shift between cooperation and parasitism (pathogenesis) during evolution, depending on the mode of symbiont transmission (vertical or horizontal), which affects the optimal strategy to increase inclusive fitness (Ewald, 1983; Herre, 1993; Bull, 1994; Sachs et al., 2004; Sachs and Wilcox, 2006). Furthermore, endosymbiosis involves various stages of development in association. The endosymbiosis between auto (photo) and heterotrophic organisms is a particularly interesting association that results in mixotrophic adaptations, which exhibits the continuity between various stages in their associations. These stages include: (i) a tentative or unstable stage, in which partners live together during parts of lifecycles or in a manner depending on their ecological conditions (Christopher and Patterson, 1983; Reisser, 1986, 1993; Smith and Douglas, 1987; Okamoto and Inouye, 2005), or hosts ingest algal cells and use their chloroplasts, a process known as kleptoplastidy (Schnepf, 1993; Gustafson et al., 2000); (ii) a stable stage, in which partners live together through their entire lifecycles and reproduce synchronously at a high rate of vertical transmission of symbionts

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(Weis, 1969, 1977); (iii) the genetically unified stage, in which many genes are moved to the partner's genome (McFadden, 2001; Howe et al., 2008). The conflict and multistage associations in endosymbioses suggest that symbiogenesis is a coevolutionary process that is driven by selection operating in participating populations of organisms as independent reproductive/survival units. Thus, unlike lateral gene transfer and recombination of genes that can occur within a single generation, endosymbiosis may evolve gradually, and not by a sudden acquisition of a complete genetic package conferring complex adaptations from partners (Law, 1991; Maynard Smith, 1991; Law and Dieckmann, 1998).

The early process of endosymbiosis, through which non-associated organisms of different species can evolve new phenotypes enabling them to associate with prospective partners, is a key to solving the problem concerning the evolutionary acquisition of adaptive phenotypes by endosymbiosis. However, the early process is an elusive concept, as it concerns a gray zone of associations that generally appear to be primitive, unstable, and variable depending on selective conditions regarding the cost-benefit balance of the association and the rates of horizontal and vertical transmission. Furthermore, associations in the early stage of endosymbiosis may obscure identifying the traits that evolved to establish the symbiosis initially. The early stage can involve one species evolving traits that benefits the other, but the latter species can also evolve the ability to exploit the former; these two cases are usually difficult to distinguish empirically. Although great effort has been devoted to determining the conditions for the evolution and stability of cooperation (Trivers, 1971; Bull and Rice, 1991; Axelrod and Hamilton, 1981; Axelrod, 1984, 1997; Axelrod and Dion, 1988; Sachs et al., 2004), the early process remains unclear. One problem is that it is methodologically difficult to analyze effectively the early process by observing natural populations. Furthermore, most theoretical models focus on a set of conditions with a limited set of strategies, and few have considered ecologically induced changes in relevant parameters involved in the early process of endosymbiosis, such as resource availability and the effect of a third species, which can affect values in the pay-off matrix.

In order to directly analyze the early evolutionary process of endosymbiosis between auto- and heterotrophic organisms, we developed an experimental ecosystem model, the CET microcosm, composed of a green alga (*Micractinium* sp., formerly described as *Chlorella vulgaris*; see Section 2), a bacterium (*Escherichia coli*), and a ciliate (*Tetrahymena thermophila*) in MC medium containing only minerals. Algae of the genera of *Chlorella* and *Micractinium*, which are generally referred to as *Chlorella*-like algae and grouped in the *Chlorella* clade, include organisms with free-living lifestyles and endosymbionts of ciliates, hydras or sponges (Lee et al., 1985; Hoshina et al., 2010; Luo et al., 2010; Pröschold et al., 2011a,b). *T. thermophila* is a bacteria-feeder that commonly lives in decaying vegetation at the mud-water interface (Doerder et al., 1995). Although there is no evidence to date that *T. thermophila* harbors algal cells in its natural habitat, a 3-year microcosm culture without transfer revealed that ciliate cells harboring the algal cells, called C-*Tetrahymena*, appeared, and increased in frequency to ca. 80–90%, which persisted throughout the course of the experiment (Nakajima et al., 2009). This increase in the frequency of C-*Tetrahymena* in the *Tetrahymena* population occurred during the period in which algal and ciliate populations declined in density, presumably due to resource depletion following the initial outbreaks of the populations in the culture. This increase in the frequency of C-*Tetrahymena* indicated that the alga and the ciliate started to interact more intimately, suggesting the possibility that the two species were evolving to form an early stage of an endosymbiotic association under the strongly resource-limited condition in the mature stage of ecosystem development in the microcosm. Following this study, Sano et al. (2009) revealed that

under bacteria-free conditions, the derived *Tetrahymena* population that was co-cultured with the derived algal population survived longer than the ancestral *Tetrahymena* that was co-cultured with the ancestral *Micractinium* population; however, it could not be determined whether the alga is benefitted by the derived *Tetrahymena* host. This result suggested that some symbiotic association between the two species was evolving in the experimental ecosystem, which enabled the *Tetrahymena* host to increase its fitness.

The mechanism for the increased longevity of the derived *Tetrahymena* population when co-cultured with the derived *Micractinium* population is currently unclear. It is evident that the alga did not evolve to become parasitic on the ciliate, as the alga did not reduce the host's overall fitness, measured in terms of longevity, in the co-culture. However, the prolonged longevity of the ciliate does not necessarily indicate that an algal cooperative trait evolved.

There are two possibilities to explain the evolution of this association. First, the alga became a slave or victim of the ciliate by evolution of the ciliate, which is here referred to as the "exploiter ciliate hypothesis." Under this scenario, the ciliate would have acquired the ability to exploit intracellular algal cells, whereas the alga would not have evolved benefiting traits. In other words, the long-term culture selected for a ciliate phenotype that exploited more photosynthates from intracellular live algal cells, or digested the algal biomass by direct killing. Although ancestral *Tetrahymena* cannot grow on the ancestral alga, the possibility remains that endo-algal cells could be digested, which is yet to be determined (Sano et al., 2009). The second possibility is that the alga itself evolved to become a cooperator, which is here referred to as the "cooperator alga hypothesis." Under this scenario, the alga would have evolved the ability to benefit the host ciliate by providing photosynthates, for example, whereas the ciliate did not improve its exploitative ability (see Appendix A for definitions of cooperator and exploiter).

In the present study, we tested the exploiter ciliate and cooperator alga hypotheses to understand a general feature of an early stage of the endosymbiosis process between auto- and heterotrophic organisms. To test the hypotheses, two-by-two combinations of the co-cultures (i.e., the survival test) were conducted between the ancestral or an isolated *Micractinium* and the ancestral or a derived clone of *Tetrahymena* to investigate the coevolutionary mechanism driving the early process of this algal-ciliate endosymbiotic association. The survival and reproductive performances of *Tetrahymena* in these cultures were measured and compared, and interspecific interactions between the alga and the ciliate were evaluated in terms of their effects on overall fitness. Certain fitness components arising from intercellular interactions were not analyzed here, such as exchanges of particular substances. Furthermore, two different algal phenotypes were evaluated in this study: one type that forms cellular aggregates in both single culture and in co-culture with *Tetrahymena*, and the other that does not form cellular aggregates under either circumstance. These phenotypes were compared because an algal cellular aggregation can affect its ingestion rate by a ciliate, and because a previous study suggested the possibility that the algal population diversified into two forms of symbiosis: endosymbiosis with *Tetrahymena* and ectosymbiosis with *E. coli* by harboring the bacterial cells within algal cell aggregates (Nakajima et al., 2009).

Conducting a factorial analysis of interactions between many isolates of both species involves a large number of combinations of co-cultures, which is a laborious task. Therefore, we first analyzed a subset of clones in detail. Specifically, two algal clones isolated from the inside of *Tetrahymena* cells in a CET microcosm on days 1820 and 1837 (the aggregate-forming SC10-1 and the non-aggregate-forming SC10-2), and two *Tetrahymena* clones (TC20 and TC4, both isolated on day 2668) were chosen for the factorial analysis. We also

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